NMR spectroscopy for Structural Biology
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Lecture: 58
Basics of solid state NMR spectroscopy - III

Good morning, so we were discussing basics of solid state NMR spectroscopy and its application in structural biology. So in previous two lectures we have discussed why solid state NMR, what kind of biological sample we can investigate. Those biological samples that are not amenable for crystallography or neither they are soluble, they are perfect for solid state NMR. For an example membrane proteins, integral membrane proteins or amyloid fibrils, these kind of difficult molecule that are that cannot be crystallized neither they can be dissolved in solution for doing liquid state NMR, they are perfect sample for solid state NMR. We also looked at how magic angle spinning improves the resolution. Then we discussed about cross polarization, how cross polarization increases the sensitivity.

Briefly, I touched upon decoupling that is required for again resolution enhancement and I said that we can use recoupling techniques for getting the lost structural information. Today I am going little more detail into these techniques and also I briefly touch upon proton detection, why it is needed and what can be done using proton detection. So let us move ahead little bit repeating what we discussed. So here is our sample packed in a rotor.

This is the solid state NMR rotor and this rotor is placed at a particular angle that is called magic angle. This is the direction of magnetic field, that's z-direction. Now this sample is spinning at very fast speed and that enhances the resolution. So essentially magic angle spinning averaged out the chemical shift anisotropy and it helps in getting good sensitivity and resolution.

So what it does? So let us take an example of a simplest molecule like glycine. So this is a simplest biological system glycine with two carbons, one CO and another CH_2. So we are getting two broad peak, one centered around 50 ppm, another centered around 170 ppm. When we start spinning this anisotropic interactions that are because of different chemical shift that we discussed last time, these spins are oriented in various direction and resultant gives you broad spectrum. So when you start spinning these are getting splitted and we are

getting many line. As we increase the spinning speed about 5 kilohertz you see we are getting two sharp line one centered around 170, one centered around 50 ppm.

At 14 kilohertz we are clearly getting to very sharp peaks, these corresponds to CO resonance, this corresponds to Cα resonance. So that is what magic angle spinning does, it average out the chemical shift anisotropy and it enhances the resolution and sensitivity. How to spin and where to spin, what we use for spinning. So as we discussed we have a different size of rotors. These rotors are made up of zirconium oxide and here we put the biological sample.

So depending upon what is the size outer diameter of these rotors we can choose and spinning speed like here 7 mm or 4 mm, 4 mm can go up to 14 kilohertz in Bruker system 3.2 mm can go up to 24 kilohertz, now 2.5 mm, this is 1.3 mm. So depending upon what is the outer diameter this rotor can go up to various speed, like this can go up to 65 kilohertz.

Now using these, people can detect proton and state of the art is 0.8 mm rotor. So that is used for proton detection, it spins really fast about 120 kilohertz. So these are the rotors where we put our biological samples and this has to be robust because we are spinning very fast.

Now for spinning, we discussed that we are using air. So there should be some caps here. So you see the caps which has a fins here. Now, here the drive pressure and bearing pressure that we are giving in the probe that helps in spinning and this floats and for detecting whether the rotor is sitting in the right slot or not, we have here the black mark that basically using optical detection system it detects and helps in spinning of the rotor. So these are the caps that are used for packing the samples, these are of different shape and size. There are two prominent, one is made up of Kel-F, one is called Vespel.

So these are two different caps that are used for packing the samples and spinning it. This rotor has to be really carefully packed, it should be balanced, it should not be leaking and caps should be tightly fitting otherwise it can break. So really one has to be careful while packing the samples and spinning. For solid materials, some tools are provided by the manufacturers. These tools helps packing the samples inside the rotors. Now where does it sit? So as I said magnetic field is in Z direction, here is dissection of the probe.

Here our rotors are sitting. So depending upon what size rotor is the whole dimension is accordingly fitting it. So this whole assembly is called a stator that is oriented at a magic angle and here we have a flexible coil connections. These are various coil for detection and for sending the pulse. Here is the place where rotor actually spins using air that comes from bottom.

So, there are two main air, bearing air and drive that actually spins here in the stator and that is create the different spinning speed that we were talking. So another term that we had discussed is decoupling that improves the resolution. So decoupling is heteronuclear decoupling. So we are exciting carbon with 90° pulse and detecting carbon, this is one dimensional spectrum. So S-spin is being detected after the decoupling irradiation with a 90° pulse.

During that detection period we are applying a high power decoupling that decouples the proton-carbon coupling that we had seen that it loosen out the interactions, so that improves the resolution. So here in a schematic I have shown you if I have a static spectrum we get really, really broad peak. When we decouple we improve little bit of resolution but with MAS we improve quite a bit of resolution and this is adamantane which has two flexible carbon and now with MAS and decoupling we can really get two sharp peak with increase in resolution as well as sensitivity.

So to reemphasize this magic angle spinning which is MAS and decoupling, these are two basic building blocks for any solid state NMR experiment that improves the resolution as well as the sensitivity. So, this is kind of like a CP pulse sequence and dipolar decoupling. So just to have an analogy, this is our 90° pulse in liquid state, we are exciting magnetization using 90° pulse and then immediately we detect it. But, in general case in solid proton dipolar coupling is huge, we cannot detect it when we are spinning at moderate speed. So for that purpose we detect mostly on the X nuclei, which is carbon.

Now carbon sensitivity you know gamma is 1/4, so therefore its sensitivity is going to be low. So what we do? We do something called cross polarization that we had discussed. So we excite with proton and by matching this condition which is called Hartman-hahn matching condition, we transfer that polarization to carbon, we detect on carbon while decoupling the proton. So this pulse sequence is called CP sequence cross polarization and

the duration for which we are cross polarizing is called τ contact time, contact time is here τ_{ct}, the duration for which we are cross polarizing it. So you know, this has to match that Hartman-Hahn condition,

$$\omega_S \gamma_S = \omega_I \gamma_I$$

So, this again improves the sensitivity of the solid state NMR experiment.

So here just I am showing it what is happening, so let us take a mixture of two solids. Now if we do direct polarization means just applying a 90° pulse here on carbon. If you just apply a 90° pulse on carbon and detect it that is called direct polarization like here what we do in liquid state. If you do direct polarization, what we are getting a broad spectrum with no resolution. But when we do cross polarization, now you can see all these peaks here, α, β in this mixture of a compound where one is negatively charged, another is positively charged, these two are mixed to make a jelly kind of substance.

If you do cross polarization, we can enhance the resolution as well as sensitivity and the enhancement in signal can be directly proportional to the ratio of their gyromagnetic constant. So this is what we achieve by doing cross polarization. So CP and MAS are building block of any solid state NMR spectrum. We achieve the sharp signal that we had discussed earlier that is needed. Needed for getting any information that is there, but we have paid a huge price.

By achieving this spectral resolution, we lost all those important interactions that were present in the solid inherently like chemical shift anisotropy, quadrapolar coupling, dipolar coupling and J-coupling. By doing this MAS and decoupling, we have lost it. This is required for a structural information. So somehow, we have to get it back, right. So this is something called you have a cake and eat it.

So we want to have a cake and eat it too. For doing that, we need to do some more trick. Decoupling is not enough, MAS is not enough, we have to do something called a recoupling. We have to recouple so that the lost important interactions are brought back.

So that is what we do. We had lots of interaction present inherently because of the spins were static. Now we force them to rotate by MAS and then by decoupling. So decoupling

all these spins become individual. Now we want to selectively introduce the interaction between them.

That is called recoupling. So here, we are recoupling it. Now by applying some trick either during spinning or applying some pulse, we need to recouple so that our interactions are brought back and this technique is called recoupling by application of various array we recouple it. So decoupling is breaking the interactions because of CSA and DD that were present inherently and recoupling is selectively reintroducing these interactions. That is what recoupling means. Now we can selectively know that which interactions is happening with which protons.

So that is what recoupling means. So how it is done? It is very simple. I have to do some trick. One of the important or essential trick is like can we do something with because there are three factors. One is RF that is given by us. One is already we are introducing spins.

So suppose we have a proton, we have two peaks here, one for Cα, one for CO. This is about 50 ppm and this is about 170 ppm. So what is the difference between these two? It is 120 ppm and suppose we are doing this experiment at say 600 megahertz. So let us see in simplistic term what we can do.

So let us take our glycine. Glycine one we have a peak coming from CH_2, one coming from CO. Here that is what we saw one around 50 ppm and one around say 170 ppm. These are the two peaks coming. So what is the difference between these two peaks? It is 120 ppm. Now suppose we are doing an experiment at say 600 MHz.

So that means 150 MHz is the resonance frequency for carbon. Now, this we are talking about 120 ppm. So how much it will be in hertz? 120 multiplied with 150, that will be something like this. So this is the difference between this. So suppose by spinning, if we introduce a rotational speed which is matching with this or half of this, then this will recouple the interaction between CO and Cα.

This is called rotary resonance condition. Now there are various other resonance conditions that we are going to talk and one of those is called REDOR recoupling. So this is the

simplest one that I talked, just by introducing the spinning speed we can recouple some of the interactions. Now there is another recoupling scheme that is called REDOR, this is recoupling for heteronuclear dipolar coupling by rotational echo double resonance.

So this is a simple scheme. So we excite with 90° pulse on proton and then we refocus using 180° pulse here and for recoupling what we do, we select the τ rotor period and we keep applying this series of 180° pulse. We have refocusing using 180° pulse in between and then we have a series of π-pulse. So this is called two phase alternating 180° RF pulse for every rotor period. Rotor period, how you can calculate? So we know the spinning speed. So for one rotor period depending upon what is the spinning speed we can calculate the one rotor period and there has to be two phase alternating 180° RF pulse for every rotor period on the carbon, on a heteronuclear channel and that basically averaged out the magic angle spinning phenomena that is there on the heteronuclear dipolar coupling.

Then we apply a 180° pulse that we had discussed about this in the middle of recoupling block on I channel that refocus the chemical shift Hamiltonian. So by doing this trick of REDOR we can reintroduce these interactions. There are various recoupling sequences that has been developed, that will be matter of extensive course on recoupling. I will not go in detail of that, I will just stick to basics of this. So some of the easy one of the recoupling sequence are called PDSD or DARR or RFDR, I will little bit discuss about these.

Essentially what we do in this recoupling sequence, we excite the proton using 90° pulse, then we transfer the magnetization using cross polarization. So now my magnetization is on carbon. Now suppose I am doing only 1D, so this t_1 and then we do something called mixing. So we can mix it by proton-driven spin-diffusion that is called PDSD or dipolar assisted rotary resonance condition. So we apply a pulse here on proton which will be half of the rotational speed or about the rotational speed that will enhance the decoupling that is done in DARR or RFDR radio frequency driven recoupling sequence.

So RFDR is again series of pulses, radio frequency driven recoupling sequence, that basically reintroduce some of the lost interactions that were introduced by MAS and decoupling and finally you acquire on carbon. So that is the recoupling sequence that is used. So now, we learn how to decouple and how to recouple. So one of the prominent proton driven spin diffusion, basically this is something called second order recoupling for polarization transfer. What we do? Basically, we have abundant of proton in the protein.

So here one amino acid, here is another amino acid. We have a Cα, Cβ, Cγ and carbonyl here. So, at the moment, we are doing only say these two nuclei. In 1D, we are detecting on carbon while using the proton magnetization for enhancement of sensitivity. So, can we transfer the polarization from proton to carbon and then detect on carbon with enhanced sensitivity. So by doing this what we are doing, we are enhancing the sensitivity and also introducing the recoupling so that we have enough magnetization. So basically, here spectral spin diffusion works on a phenomena called exchange of longitudinal magnetization.

So what we do here? We polarize the magnetization from proton to carbon and then we decouple proton and then let them mix, that we have seen in typical NOESY spectrum. We put it in the longitudinal magnetization and let the magnetization mix, so that is what happened here. But here, the magnetization is mixing through dipolar coupling and this is happening by something called flip-flop mechanism. So using this we can utilize the magnetization transfer in homonuclear correlation experiment.

So here we are decoupling protons and letting them mix. So here is the mixing happening and then while decoupling proton we detect on carbon. So this is called PDSD, proton driven spin diffusion. By method of spin diffusion magnetization is getting transferred and we are detecting on carbon. Now this can come into 2D version as well. So here if we introduce a t_1 evolution time, that we have seen liquid state that will be converted into t_2.

So just to remind you how we do 2D, it is a simple experiment, more than one pulse we need two pulse. So here in 2D experiment, you do series of 1D experiment. In 1D experiment what we have done? So we started with a proton and then we were detecting it. So this was two pulse experiment and in the second experiment we are increasing this distance between these two pulse.

In third experiment, we were further increasing this distance. So similar thing we are doing here into solid state as well. We are starting with a preparation stage, and then there is a mixing stage, and then we are detecting it. In the second experiment, we are increasing the distance between this preparation stage and mixing stage, and in the third experiment even more, fourth experiment even more, fifth experiment again. So by increasing this time distance, we are creating another time domain where spin is evolving.

Then we are detecting in the direct dimension, so this is called indirect dimension, this is called direct dimension. So if you write it your ω_2, which we are detecting with a time this changes, you can see the peaks is changing and now we can do Fourier transform of these two, ω_2 direct dimension and ω_1 indirect dimension shows a correlation peaks that we have seen. So this kind of concept is again employed in solid state for doing the 2D and that is what I showed you. So start from proton and cross polarize on carbon and then we are introducing this time evolution. During that time we are decoupling it and then here we are allowing the spins to mix either by themselves which is called PDSD or by application of RF pulse which is called DARR.

So by doing these we are introducing recoupling while these spins are mixing and then we finally detect while decoupling protons. So that is what is the 2D. Now we can establish the carbon-carbon correlation spectrum using PDSD or DARR experiment and that is what happens. So let us sum up what we have learnt till now.

In solid state we have to take a rotor. That rotor should containing all the biological sample whether it is materials or amyloid fibres or membrane protein. We pack them in the rotors, we put the cap tightly, then we put in the magnet, in the magnet it is oriented at a magic angle at 54.7°, and then we start spinning at desired spinning state. So with MAS and then we are applying decoupling sequences, we are getting sharp lines for each spins.

Now we can extend this 1D information into 2D where establish the correlations. So this is now 2D correlation but here you see diagonal is only appearing, so this is self-correlating. When we introduce this recoupling using RF sequence or PDSD, so decoupling increasing the sensitivity and resolution, recoupling introducing again the interactions between these spins.

So you can selectively introduce it. Now you can see these two spins are correlating. So this is the 2D with correlation matrix. So that is what we are achieving. Spinning speed, cross polarization removes the anisotropy, and removes the strong coupling. It increase the spectral resolution and finally using recoupling sequence, we are extracting the spectral information. So using these things now we have transition from one dimensional solid state NMR to two dimensional which is the essential for getting any structural information.

So let us see how our spectrum looks like. 2D correlation spectrum, so like here PDSD or DARR is carbon-carbon correlation spectrum. You see it looks like exactly like a NOESY spectrum that we have seen in liquid state NMR. This looks like HSQC spectrum, this is NC correlation. So let me just explain you briefly what is PDSD or NC.

So here, we have N and Cα. So this is the correlation we have. So here we have started with a proton, transferred to carbon and then we are using the again CP condition, we are detecting on carbon, while decoupling protons. The one dimension, the direct dimension is N evolution and then indirect dimension is a carbon evolution. So that is what we are having here, the carbon and nitrogen correlation. Now each of these peaks, so if you are doing NCα, each of these peaks giving idea about one amino acid.

So each amino acid will have one NCα correlation. So this is exactly like HSQC spectrum that we have seen in the liquid state. That is the NC. Now carbon-carbon spectrum PDSD, so what we are doing? We are first polarizing all protons, then using CP we are transferring to carbons, these protons can transfer the magnetization to neighboring carbon or directly attached carbon, and then we are mixing them, so carbon mixing is happening and therefore you see lots of peaks are coming like NOESY depending upon distance. So if you say take any isolated peak, here are threonine peak, these are serine peaks.

So you can see Cα-Cβ peak is getting correlated. Here Cα-Cγ peaks are correlated. Here serine has only two carbon Cα-Cβ, so that is coming here. These all are actually the γ and δ of isoleucine that are there. So in the PDSD, depending upon how long mixing time we are keeping, how much dark power we are applying, we can achieve longer correlation. So, all the way from Cα to Cβ to Cγ to Cδ even the neighboring one, like some of these will be neighboring interactions and these interactions like NOESY spectrum are very useful for getting the resonance assignment, also getting the structural constraints.

So just some more spectrum, so we can have a HSQC kind of a spectrum. So in this experiment what we have done here that we started with a proton, transfer to nitrogen. So like HSQC we had earlier, we started from proton, transfer to nitrogen and then in indirect dimension we are evolving proton while detecting on nitrogen. So here we are getting ^{1}H-^{15}N spectrum, but things to be noticed here very importantly, protons still has a broader line width.

You look at the line width, this looks really elongated and if we compare this with NC spectrum, this is quite well resolved. So proton at the moderate speed still has lots of inherent problem of spectral overlap because the dipolar couplings are not averaged out but still we are getting quite decent spectrum in the solid. However if you look at the line width is quite huge about 250 hertz. Similarly we can do ^{13}C-^{15}N spectrum like ^{13}C-HSQC.

Similarly here we can do HSQC but again the lines are going to be broad. So here if you look at this is Cα and Hα correlation but line width are really broad. So we are not getting enough resolution here and it will be difficult to assign. Few of the peaks you can see here are still sharp, these are coming from Cα, Hα and these can be easily identified. Here even though proton is in indirect dimension because the dipolar coupling could not be averaged out we are really getting broad spectrum and therefore the faster and faster speeds are required and lots of technological development happening in this direction. But at a moderate speed, heteronuclear detections and heteronuclear correlations are of quite benefit. Here we are almost getting liquid like spectral resolution using PDSD and using NCA experiment we can essentially get the resonance assignment.

So people have developed various sequential resonance assignment like whatever we have in liquid, just protons is removed in the name. So you can have HNCACO like it starts from proton transfer to nitrogen and then to Cα-CO like these are the various transfer schemes, NCOCA this is corresponding to HNCOCA. H is not involved here, CaNCO, NCACB, NCOCB, CaNCOCB. So you can transfer the magnetization from one nuclei to another nuclei to establish various correlations between ^{15}N, Cα, Cβ, CO and that is what helps us in doing the resonance assignments in solid. So, what I am going to do in the next class, I will be taking some of those methods how to go for resonance assignment, but to give you an impression that if we assign all those peaks, we can easily get the secondary structural chemical information. Like in liquid, we have seen that depending upon what is the resonance frequency for Cα and Cβ, we can find it out whether they are up field shifted or down field shifted.

Like suppose we have a β-sheet from the random coil the Cα will be up field shifted and in α-helix it will be down field shifted, Cβ will be reversed. So we can assign those and then subtract the random coil from these value, plot along the sequence and we can find it out the secondary structural information of the proteins or polypeptide chain. So you see by doing these experiments, we are arriving at the secondary structural information using

solid state NMR techniques. So here I will stop by giving you impression that solid state NMR can be used for structural determination. I will go little more detail, show you what are the pulse sequence in details, how you can use those strategies for resonance assignment in solid and how you can get the structural parameters for getting the structural information as well as the dynamic information in next classes. Thank you very much.